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Site Specific Incorporation of Amino Acid Analogues Into Proteins *In Vivo.*	DAAD 19-99-1-0300
6. AUTHOR(S) Uttam L. RajBhandary	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts Institute of Technology 77 Massachusetts Avenue Cambridge, MA 02139 USA	8. PERFORMING ORGANIZATION REPORT NUMBER
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11. SUPPLEMENTARY NOTES

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13. ABSTRACT (Maximum 200 words)

Our objective is to develop general methods for the site-specific incorporation of amino acid analogues into proteins in bacterial and in eukaryotic cells. The approach consists of the use of an amber suppressor transfer RNA (tRNA) aminoacylated with an amino acid analogue, with the help of a mutant aminoacyl-tRNA synthetase, to insert the amino acid analogue at a specific site in a protein. The site of insertion of the analogue is specified by an appropriately placed amber termination codon within the gene for the protein of interest. This approach has two key requirements: (1) an amber suppressor tRNA, which can not be aminoacylated by any of the endogenous aminoacyl-tRNA synthetases and (2) an aminoacyl-tRNA synthetase, which aminoacylates the amber suppressor tRNA but no other tRNA in the cell. Therefore, an important first goal is to identify such a 21st aminoacyl-tRNA synthetase-tRNA synthetase-amber tRNA pair. This goal has been achieved. Several mutations have been introduced into yeast tyrosyl-tRNA synthetase (TyrRS) for isolating mutants that incorporate iodotyrosine into tRNA instead of tyrosine. Work on an alternative approach applicable to mammalian cells has led to a possible general approach for the introduction of two different amino acid analogues into a protein.

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5. Summary of Results

21st Aminoacyl-tRNA Synthetase – Suppressor tRNA Pairs

Previously, we described the identifications of two 21st synthetase suppressor tRNA pairs, one for use in eukaryotes and the other for use in eubacteria. These 21st-synthetase-tRNA pairs were (i) yeast-tyrosyl-tRNA synthetase (TyrRS) in conjunction with an amber suppressor tRNA derived from *E. coli* initiator formylmethionine tRNA and (ii) *E. coli* glutaminyl-tRNA synthetase (GlnRS) in conjunction with an amber suppressor tRNA derived from human initiator tRNA (1). The next step is isolation of mutants in the yeast TyrRS gene and the *E. coli* GlnRS such that they aminoacylate the suppressor tRNA with the amino acid analogues of choice instead of the normal amino acid. This work continues to be part of our long-term effort. The focus of work in the last year has been to isolate mutants of yeast TyrRS that incorporate iodotyrosine into tRNA instead of tyrosine. Mutants Tyr43 to Val and Gln186 to Cys have been made. Other mutants Asp177 to Asp321 to Arg and the double mutant His81 to Ala, Asp177 to Thr have also been made and the mutant proteins are being tested for their activity in activating iodotyrosine and attaching it to the amber suppressor tRNAs.

Import of Suppressor tRNAs into Mammalian COS1 Cells

The above approach using 21st synthetase-tRNA pairs requires the isolation, one at a time, of mutants in the 21st aminoacyl-tRNA synthetase (aaRS), which activate the amino acid analogues and attach it to the suppressor tRNA instead of the normal amino acid. A further requirement is that the amino acid analogue should be readily imported into the organism of choice. An alternative approach that does not require a mutant aaRS and that has the potential of being generally applicable for a number of purposes would be the import into cells of suppressor tRNAs chemically aminoacylated with the amino acid analogue of choice. This method also does not rely on uptake of the desired amino acid analogue from the medium into cells. The only requirement is that the suppressor tRNA must not be aminoacylated by any of the aaRSs in the cell.

Previously, we identified the conditions necessary for the import of amber and ochre (UAA) suppressor tRNAs derived from *E. coli* initiator $tRNA_2^{fMet}$ into mammalian COS1 cells and showed that these suppressor tRNAs are active in the suppression of amber and ochre codons in a reporter mRNA. We further showed that an aminoacylated amber suppressor tRNA (*supF*) (Figure 1A) derived from the *E. coli* tyrosine tRNA can be imported into COS1 cells and acts as a suppressor of amber codons, whereas the same suppressor tRNA imported without prior aminoacylation does not, suggesting that the *supF* tRNA is not a substrate for any mammalian aminoacyl-tRNA synthetase. These results allow the use of the *supF* tRNA aminoacylated with an amino acid analogue as a general approach for the site-specific insertion of a variety of amino acid analogues into proteins in mammalian cells (2).

Essentially, all of the *in vitro* and *in vivo* work with unnatural amino acid mutagenesis has, so far, involved the use of amber suppressor tRNA along with an amber codon at the site of interest in the protein gene. The availability of another suppressor tRNA/nonsense codon pair would greatly add to the versatility of unnatural amino acid mutagenesis. We have now shown that an ochre suppressor tRNA derived from *E. coli* tyrosine tRNA (Figure 1B) is also not aminoacylated by any of the mammalian aaRSs (3). Import of aminoacylated ochre suppressor tRNA leads to suppression of an ochre codon in a firefly luciferase reporter gene, whereas import of the same tRNA without prior aminoacylation does not (Figure 2 and Table 1). Thus, the ochre suppressor tRNA can be used to specifically suppress an ochre codon.

Concomitant Suppression of Amber and Ochre Codons in a mRNA

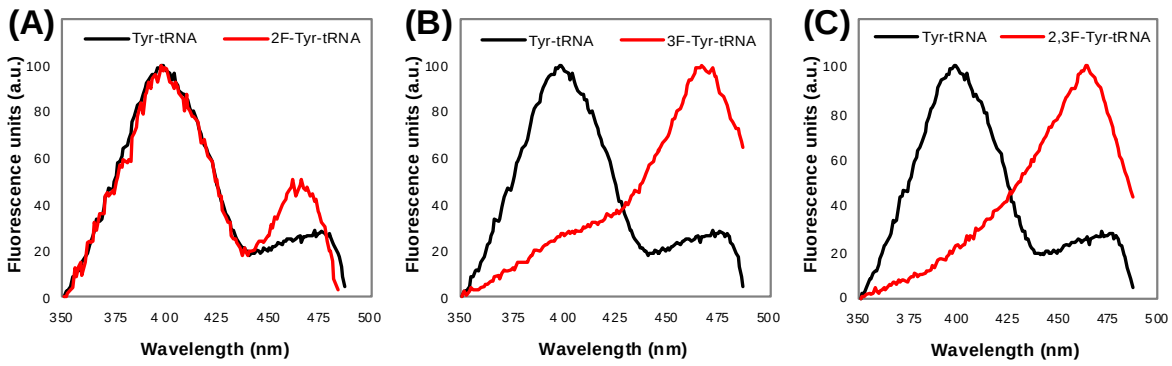
The availability of amber and ochre suppressor tRNAs that are not aminoacylated by mammalian aaRSs opens up the possibility of site-specific insertion of two different unnatural amino acid into a protein (Figure 3). This possibility adds greatly to the application of unnatural amino acid mutagenesis to problems in contemporary biology. For example, introduction of two different fluorescent amino acids into a reporter protein would allow the use of fluorescence resonance energy transfer (FRET) to study protein conformation and dynamics in mammalian cells *in vivo*. Such fluorescent amino acids could thus be used as biosensors. Similarly, site-specific insertion of phospho-amino acids such as phosphothreonine and phosphotyrosine could be used to specifically activate one of the many mitogen activated protein (MAP) kinases in a cell in the absence of an extracellular or upstream signal. Such studies could provide important information on the relationship between each of the MAP kinases and the transcription factors that they phosphorylate and the genes that they activate.

In studies along these lines, we have shown that import of a mixture of aminoacylated ochre and amber suppressor tRNAs leads to concomitant suppression of an ochre and an amber codon in the firefly luciferase gene (Table 2). This result provides a general approach for site-specific insertion of two different unnatural amino acids into proteins in mammalian cells (3). To the best of our knowledge, this result also represents the only example of suppression *in vivo* of two different termination codons in a single mRNA.

Assay for Site-specific Insertion of an Amino Acid Analogue into Protein in Mammalian Cells

As a test case, we will use the site-specific insertion of 2,3-difluorotyrosine into a position normally occupied by tyrosine 66 in the green fluorescent protein (GFP). The chromophore in GFP is formed from a sequence of three contiguous amino acids Ser65-Tyr66-Gly67 and the excitation and emission spectra of GFP are quite sensitive to changes in tyrosine 66. Replacement of Tyr66 by Trp, Phe or His leads to significant changes in the excitation and emission spectra. It is, therefore, likely that a change from tyrosine to 2,3-difluorotyrosine will also lead to a significant shift that can be detected in crude extracts without needing purification of the GFP. We have introduced an amber mutation at the codon corresponding to Tyr66 (*GFPam66*) and shown by using confocal fluorescence microscopy that this amber codon can be suppressed in mammalian cells by import of an amber suppressor tRNA. Therefore, as first evidence that an amino acid analogue can be inserted at specific sites in a protein in mammalian cells using tRNA import, we will aminoacylate the *SupF* amber suppressor tRNA with 2,3-difluorotyrosine and import it into COS1 cells. Aminoacylation of the suppressor tRNA with 2,3-difluorotyrosine can be carried out with *E. coli* TyrRS, which uses fluorotyrosines as substrate. Extracts of mammalian cells transfected with the *GFPam66* mutant gene and the 2,3-difluoroTyr-suppressor tRNA will be used for the spectral characterization of the GFP mutant. If necessary, fluorescence activated cell sorting will be used for further enrichment of cells producing the mutant GFP. Caroline Köhrer in the laboratory has shown that crude extracts of mammalian cells transfected with plasmids carrying the GFP gene can be used directly for recording of fluorescence spectra. Additional confirmation of the incorporation of 2,3-difluorotyrosine into GFP will be obtained by His-tagging the protein, followed by its purification and mass spectral analysis.

As a control *in vitro* for the above experiment, Caroline Köhrer has shown that GFP in which Tyr66 is replaced by 2-fluoro, 3-fluoro and 2,3-difluorotyrosine displays a shift in the excitation maximum from 395-400 nm to 465-470 nm. (The extent of the shift parallels the pKa of the fluorotyrosine, with the 2,3-difluorotyrosine showing the clearest shift.



2-fluorotyrosine
pK_a = 9.0

3-fluorotyrosine
pK_a = 8.4

2,3-difluorotyrosine
pK_a = 7.6

Excitation spectra of GFP synthesized in an *E. coli in vitro* transcription/translation system. The reactions contained pGFPam66 DNA and amber suppressor tRNA aminoacylated with 2F-Tyr **(A)**, 3F-Tyr **(B)**, and 2,3F-Tyr **(C)**. The excitation spectrum of GFP with tyrosine at position 66 is shown for comparison.

Attempts at Use of *In Vitro* Transcripts of Amber Suppressor tRNAs

The approach involving import of aminoacylated suppressor tRNA into mammalian cells requires the purification of substantial amounts of *E. coli* SupF amber suppressor tRNA. While this has not been a problem so far, we have also generated *in vitro* transcripts of the same suppressor tRNA using T7 RNA polymerase. This approach has the potential of providing virtually unlimited amounts of purified amber suppressor tRNAs and is much less labor intensive. Because the *in vitro* transcript lacks all base modifications and the modification of A at position 37 next to the anticodon sequence is known to be particularly important for suppressor activity of tRNA, we have introduced this base modification *in vitro* using the enzyme tRNA isopentenyl transferase. The gene for the enzyme was cloned and the enzyme purified as a his-tagged protein. Preliminary results show that the suppressor tRNA carrying the isopentenyl modification is much more active in cells free translation systems compared to the *in vitro* transcript lacking the base modification.

Suppression of Human Genetic Diseases Caused by Nonsense Mutations

Nonsense mutations are often responsible for a variety of human genetic diseases. The successful import of suppressor tRNAs into cell lines and evidence for their function in suppression raises the question of whether tRNA import can be used as a possible treatment for some of the genetic diseases. In attempts to study this, we have obtained fibroblast (GM03055) and lymphoid cell lines (GM07732) isolated respectively from patients suffering from *Xeroderma pigmentosum* and cystic fibrosis. The fibroblast cell line has an ochre mutation in the hRAD30 gene coding for a DNA polymerase that can bypass thymine-thymine dimers induced by UV damage of DNA. Defects in hRAD30 interfere with error-free replication of UV-lesions and lead to *Xeroderma pigmentosum*. As a first step in these studies, we have used the highly sensitive luciferase reporter gene to determine the reagents and conditions optimal for transfection of nucleic acids into the fibroblast cell line. We have found that in contrast to COS1 or HEK293 cells, polyethylene imine is the best reagent for transfection of fibroblasts (Table 3). We have obtained small amounts of antibodies raised against peptide epitopes of hRAD30 from another laboratory and are planning to have more of the antibodies made. The antibodies will be used as reagents to see whether the ochre mutation in the hRAD30 gene in the fibroblast cell line (GM03055) can be suppressed by the import of an ochre suppressor tRNA.

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List of Publications

(a) Papers Published in Peer-reviewed journals:

A. K. Kowal, C. Köhrer and U. L. RajBhandary. Twenty-first aminoacyl-tRNA synthetase-suppressor tRNA pairs for possible use in site-specific Incorporation of amino acid analogues into proteins in eukaryotes and in eubacteria. *Proc. Natl. Acad. Sci. USA* **98**, 2268-2273 (2001).

C. Köhrer, L. Xie, S. Kellerer, U. Varshney and U.L. RajBhandary. Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins. *Proc. Natl. Acad. Sci. USA* **98**, 14310-14315 (2001).

C. Köhrer, J-H. Yoo, M. Bennett, J. Schaack and U. L. RajBhandary. A possible approach to site-specific insertion of two different unnatural amino acids into proteins in mammalian cells using nonsense suppression. *Chem. and Biol.* **10**, 1095-1102 (2003).

(b) Papers Published in Non-peer-reviewed Journals or in Conference Proceedings:

C. Köhrer and U. L. RajBhandary. Proteins carrying one or more unnatural amino acids. *In* The Aminoacyl-tRNA Synthetases. Eds. Ibba, Cusack and Franklin. In press, to be published by Landes Biosciences Publishers, Chapter 33, (2004).

(c) Abstracts:

International tRNA meeting 2000, Cambridge, UK. Potential 21st synthetase-tRNA pairs for use in eubacteria and in eukaryotes. Talk presented by Anne Kowal.

Asilomar Conference 2002, Asilomar, CA. Import of suppressor tRNA into mammalian cells: A possible approach to site-specific insertion of amino acid analogues into proteins *in vivo*. Talk presented by Uttam RajBhandary.

American Society of Microbiology meeting 2003, Washington DC. From Initiator tRNAs to Proteins Carrying Unnatural Amino Acids. Talk presented by Uttam RajBhandary.

International tRNA World meeting 2003, Banz, Germany. Concomitant suppression of amber and ochre codons in a single mRNA in mammalian cells: A general approach to site-specific insertion of two different unnatural amino acids into proteins. Talk presented by Caroline Köhrer.

Invitrogen meeting 2003, San Diego, CA. Mutant tRNAs and proteins carrying unnatural amino acids in eukaryotic cells. Talk presented by Uttam RajBhandary.

6. List of scientific personnel earning advanced degree while employed on this project

None.

7. Report of Inventions

Patent application filed. Title of invention: "Suppressor tRNA Systems in Mammalian Cells for the Introduction of Unnatural Amino Acids in Polypeptides."

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Bibliography

1. Twenty-first aminoacyl-tRNA synthetase-suppressor tRNA pairs for possible use in site-specific incorporation of amino acid analogues into proteins in eukaryotes and in eubacteria. A. K. Kowal, C. Köhrer and U. L. RajBhandary. *Proc. Natl. Acad. Sci. USA* **98**, 2268-2273 (2001).

2. Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins. C. Köhrer, L. Xie, S. Kellerer, U. Varshney and U.L. RajBhandary. *Proc. Natl. Acad. Sci. USA* **98**, 14310-14315 (2001).

3. A possible approach to site-specific insertion of two different unnatural amino acids into proteins in mammalian cells using nonsense suppression. C. Köhrer, J-H. Yoo, M. Bennett, J. Schaack and U. L. RajBhandary, *Chem. and Biol.* **10**, 1095-1102 (2003).

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Appendixes (Illustrations and Tables)

Figure 1. Cloverleaf structures of amber and ochre suppressor tRNAs derived from the *E. coli* $tRNA^{Tyr}$. Arrows indicate the changes in the suppressor tRNAs; (A) amber suppressor tRNA *supF*; (B) ochre suppressor tRNA *supC*.A32.

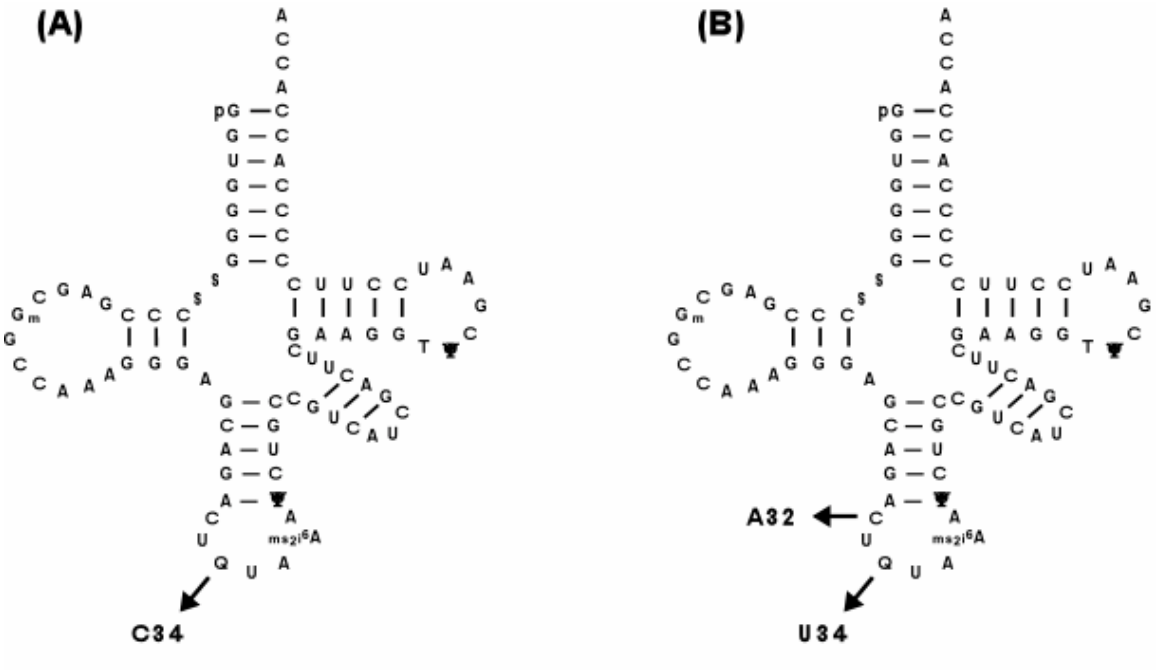

Figure 2. Schematic representation of the luciferase reporter gene encoding a *Renilla* luciferase/firefly luciferase (RLuc/FLuc) fusion protein under the control of the P_{MMSV} promoter. The stop mutations in the firefly luciferase gene are indicated; P_{MMSV}, Moloney Murine Sarcoma Virus LTR promoter.

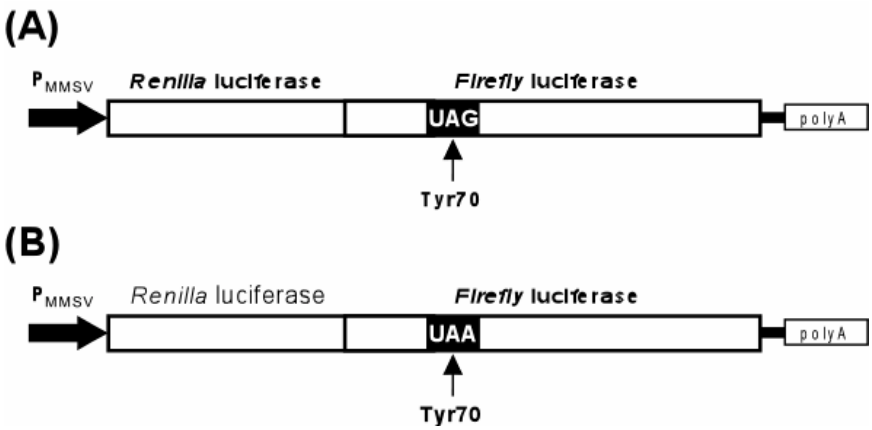

Figure 3. Scheme for concomitant suppression of amber and ochre stop codons in the firefly luciferase mRNA. U*, modified uridine.

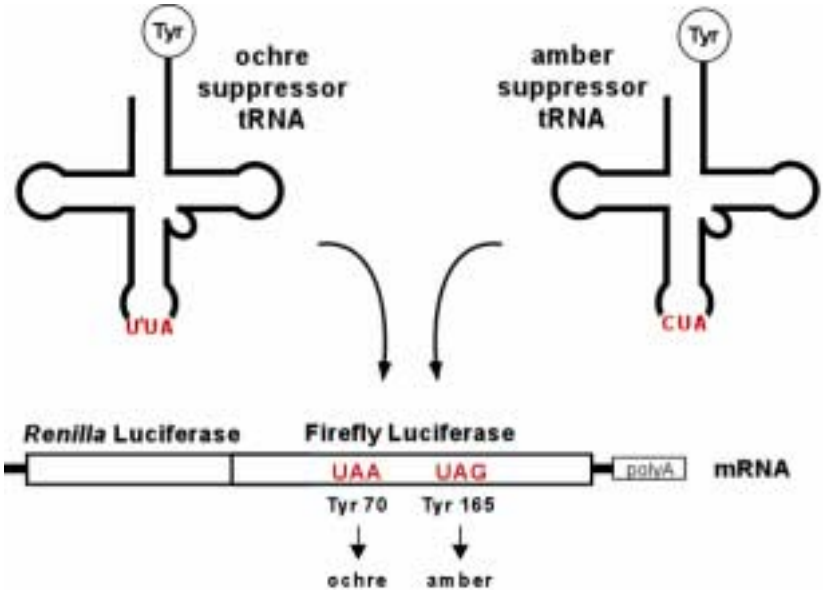

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Table 1. Import of *supF* and *supC*.A32 tRNA into HEK293 luciferase cell lines. HEK293-F22 (*oc*) cells were transfected with *supF* and *supC*.A32 tRNA with and without prior aminoacylation as indicated. Luciferase activities were measured in total protein extracts and given as RLU (Relative Luminescence Units) per μg of protein.

	Suppressor tRNA[(a)]	FLuc activity x 10^3 (RLU/μg)	Relative FLuc activity
1	*supC*.A32	0.4 ± 0.02	0.8 %
2	Tyr-*supC*.A32	50.9 ± 2.93	100 %
3	*supF*	0.01 ± 0.02	< 0.1 %
4	Tyr-*supF*	0.01 ± 0.02	< 0.1 %
5	mock	0.01 ± 0.01	< 0.1 %

Table 2. Import of suppressor tRNAs into human embryonic kidney (HEK293) cells. The HEK293-D9 (oc70am165) luciferase cell line was transfected with a mixture of amber and ochre suppressor tRNAs (*SupF* and *SupC*) derived from *E. coli* tyrosine tRNA. *E. coli* initiator tRNA wild type (tRNAwt) was used to keep the amount of tRNA and transfection conditions constant. Luciferase activities were measured in total protein extracts and are given as RLU (Relative Luminescence Units) per μg of protein.

[(a)]HEK293 cells were transfected with 3.75 μg of active suppressor tRNA as indicated. $tRNA^{fMet}$ was added to keep the amount of total tRNA constant throughout the experiment.

***SupF* (amber)**	***SupC* (ochre)**	**tRNAwt**	**Firefly luciferase activity x 1000 (RLU FLuc/μg)**
-	-	+	0.05
+	+	-	47.8
+	-	+	0.1
-	+	+	1.6

Table 3. Transfection of human fibroblasts (cell line GM03055) using various transfection reagents. Fibroblasts were transfected with 2 μg of plasmid pLNCX RLucFLuc (wt). Luciferase activities were measured in total protein extracts and are given as RLU (Relative Luminescence Units) per μg of protein.

Transfection reagent	***Firefly* luciferase activity x 1000 (RLU FLuc/μg)**
Effectene[(1)]	16.0
Lipofectamine Plus[(2)]	37.7
DMRIE-C[(2)]	1.5
Lipofectin[(2)]	0.1
Polyethyleneimine[(3)]	271.4

[(1)]Qiagen; [(2)]Invitrogen/Gibco; [(3)]MBI Fermentas

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Uttam RajBhandary

Uttam L. RajBhandary
Lester Wolfe Professor of Molecular Biology
Biology Department, 686-671
Massachusetts Institute of Technology
Cambridge, MA 02139